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## DESCRIPTION

### Monoclonal Antibodies against Human Apoptosis Inhibitory Protein NAIP and Method for Assaying The NAIP

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*Art Al.*

#### Technical Field

The present invention relates to monoclonal antibodies, which  
10 specifically recognize Human Apoptosis Inhibitory Protein NAIP and an  
immunoassay method of the NAIP.

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#### Background Art

Apoptosis is a type of programmed death of a cell, in which  
phenomena such as lack of contact with the surrounding cells, inspissation  
of cytoplasm, aggregation of chromatin and karyopyknosis related to the  
activity of endonuclease, fragmentation of nucleus, the cell being changed  
20 into membrane-wrapped bulboid corpuscles, englobement of the bulboid  
corpuscles by the adjacent macrophage or epithelial cells, or fragmentation of  
the nucleosome unit of DNA into DNA fragments of 180-200 base length by  
the activity of endonuclease are observed. Apoptosis has been discussed as  
a mechanism in which the final fragments of apoptic somatic cells exhibiting  
25 the aforementioned phenomena are englobed by the adjacent cells (e.g.,  
"Immunology Today", 7:115-119. 1986; Science 245:301-305. 1989).

As the gene that controls the apoptosis described above, the bcl-2  
gene, which is one of oncogene discovered from B cell lymphoma in 1985, is  
30 known. This bcl-2 gene appears quite frequently in cells of the immune  
system or neuronal cells. It is assumed that the substance produced as a  
result of expression of the gene suppresses the apoptosis of such cells,

whereby the function of the human immune and neuronal systems are constantly maintained the homeostasis thereof. In addition, as the bcl-2 gene appears in a fetus in an especially wide range, it is assumed that the gene plays an important role in morphogeny during the ontogenic process.

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On the other hand, the inventors of the present application have isolated Neuronal Apoptosis Inhibitory Protein (NAIP) gene, as the gene causing Spinal Muscular Atrophy (SMA) which is a familial genetic disease, from the human chromosome 5q13.1 domain (Roy et al., Cell 80: 167-178, 1995), and they have filed a patent application for the gene (PCT/CA95/00581). Specifically, it is considered that the mutation of the NAIP gene or the decrease in the number of copies therefrom causes apoptosis of spinal neurons, resulting in the development of SMA. Further, in a case in which the NAIP gene is introduced into various cultured cells and stimulation is provided to the cells to induce apoptosis, it has been found out that the death of the cells is significantly prevented (Liston et al., "Nature" 379: 349-353, 1996). In this case, it has also been found out that NAIP functions as the apoptosis inhibitory factor not only to the neuronal cells but also to the somatic cells as a whole.

The inventors of the present application have isolated the full amino acid sequence of NAIP and cDNA encoding the NAIP, and filed a patent application thereof (Japanese Patent Application No. 9-280831).

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As described above, NAIP is a protein which is concerned with various apoptosis-related diseases including SMA. In order to understand the mechanism of a patient's developing such diseases, diagnose the risk for developing the diseases, prevent the development of the diseases or reduce the severity of the diseases, and develop the medical technique and medicines for treatment, it is essential to accurately assay the amount of expressed NAIP.

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The inventions of the present application has been contrived in consideration of the aforementioned task, and objects of the present invention is to provide anti-NAIP monoclonal antibodies, which are essential for assaying NAIP and a NAIP assaying method using the monoclonal antibodies.

#### **Disclosure of Invention**

10 The inventors of the present application have assiduously studied the solution of the aforementioned task, and as a result, discovered that the epitopes of NAIP exist in the amino acids of the 256-586th and the 841-1052nd in SEQ ID NO: 1.

DRAFT EDITION 00000000000000000000000000000000

15 The present application, on the basis of the discovery, provides an anti-NAIP monoclonal antibody recognizing a human apoptosis inhibitory protein NAIP having the amino acid sequence of SEQ ID NO: 1, which is produced from hybridoma prepared by fusing a myeloma cell line with antibody-producing cell of mammal immunized by antigen containing a polypeptide which comprises amino acid sequence of the 256-586th, the 841-1052nd or parts thereof in SEQ ID NO:1

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The present application provides, as specific embodiments of the monoclonal antibody: anti-NAIP monoclonal antibody hnmc365, which is produced from hybridoma 656-1 (FERM BP-6919), and its epitope is the 354-365th region in SEQ ID NO: 1.; anti-NAIP monoclonal antibody hnmc381, which is produced from hybridoma 656-2 (FERM BP-6920), and its epitope is the 373-387th region in SEQ ID NO: 1; and anti-NAIP monoclonal antibody hnmc841, which is produced from hybridoma 841 (FERM BP-6921), and its epitope is the 841-1052nd region in SEQ ID NO:1.

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The present application provides the first method of assaying NAIP,

which comprises contacting a marker-labeled anti-NAIP monoclonal antibody with a sample containing NAIP thereby binding the marker-labeled antibody with NAIP, and measuring signal strength of the marker in the bound structure.

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In the first assay method, it is preferred that the anti-NAIP monoclonal antibody is any one of said hnmc365, hmnc381 and hmnc841, and that the marker is an enzyme, a radioactive isotope or a fluorescent colorant.

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The present application provides the second method of assaying NAIP which comprises contacting an anti-NAIP primary antibody with a sample containing NAIP thereby binding the primary antibody with NAIP, further binding the bound structure with an anti-NAIP secondary antibody, and measuring signal strength of a marker bound with the secondary antibody, wherein:

- (1) the primary antibody and the secondary antibody are both said anti-NAIP monoclonal antibody;
- (2) the primary antibody is said anti-NAIP monoclonal antibody and the secondary antibody is an anti-NAIP polyclonal antibody; or
- (3) the primary antibody is an anti-NAIP polyclonal antibody and the secondary antibody is said anti-NAIP monoclonal antibody.

In the second assay method, it is preferred that the primary antibody is immobilized on solid phase, that the anti-NAIP monoclonal antibody is any one of said hnmc365, hmnc381 and hmnc841, and that the marker is an enzyme, a radioactive isotope or a fluorescent colorant.

The present application provides the first kit for assaying NAIP at least including:

- (a) a plate on which an anti-NAIP primary antibody is immobilized; and
- (b) an anti-NAIP secondary antibody labeled with a marker, wherein:

- (1) the primary antibody and the secondary antibody are both said anti-NAIP monoclonal antibody;
- (2) the primary antibody is said anti-NAIP monoclonal antibody and the secondary antibody is an anti-NAIP polyclonal antibody; or
- 5 (3) the primary antibody is an anti-NAIP polyclonal antibody and the secondary antibody is said anti-NAIP monoclonal antibody.

In the first assay kit, it is preferred that the marker is a radioactive isotope, a fluorescent colorant or an enzyme, and in the case of the marker being emzyme the kit further includes:

- 10 (c) a substrate which develops a color by the enzyme activity.

The present invention provides the second kit for assaying NAIP at least including:

- 15 (a) a plate on which an anti-NAIP primary antibody is immobilized;
- (b) an anti-NAIP secondary antibody; and
- (c) a marker to be bound with the secondary antibody, wherein:
- (1) the primary antibody and the secondary antibody are both said anti-NAIP monoclonal antibody;
- 20 (2) the primary antibody is said anti-NAIP monoclonal antibody and the secondary antibody is an anti-NAIP polyclonal antibody; or
- (3) the primary antibody is an anti-NAIP polyclonal antibody and the secondary antibody is said anti-NAIP monoclonal antibody.

25 In the second assay kit, it is preferred that the marker is a radioactive isotope, a fluorescent colorant or an enzyme, and in the case of the marker being emzyme the kit further includes:

- (c) a substrate which develops a color by the enzyme activity.

30 In said assay kits, it is further preferred that the anti-NAIP monoclonal antibody is any one of said hmnc365, hmnc381 and hmnc841

### Brief Description of Drawings

Fig. 1 is a graph that shows a relationship between the concentration  
5 of purified NAIP in a sample solution and the absorbance measured by the  
method described in Examples.

Fig. 2 shows the results of Western Blotting in which anti-NAIP  
10 antibodies of a mononuclear cell solution derived from human peripheral  
blood were used. Lanes represent, in order, 1. monoclonal antibody  
hnmc365; 2. monoclonal antibody hnmc381; 3. monoclonal antibody  
hnmc841; and 4. polyclonal antibody. The concentrations of the antibodies  
each resulted from dilution by 250 times.

### Best Mode for Carrying Out the Invention

The anti-NAIP monoclonal antibodies of the present invention may be  
produced by the following steps, for example, according to the known method  
20 ("Monoclonal Antibody" Takaaki NAGAMUNE and Hiroshi TERADA, Hirokawa  
Shoten, 1990; "Monoclonal Antibody" James W. Goding, third edition,  
Academic Press, 1996).

#### 1. Preparation of hybridomas

25 A mammal animal is immunized by using an immunogen containing  
a polypeptide, the polypeptide comprising amino acid sequence of the 256-  
586th, the 841-1052nd or parts thereof in SEQ ID NO: 1. An additional  
immunization is optionally carried out according to necessity so that the  
animal is sufficiently immunized. Next, the antibody-producing cells  
30 (lymphatic cells or spleen cells) are isolated from the animals and fused cells  
are obtained by fusing the antibody-producing cells and myeloma cells. A  
plurality of cells that respectively produce the targeted monoclonal antibody

are selected and cultured, thereby obtaining hybridomas. The steps for the procedure will be each described in detail hereinafter.

a) Preparation of immunogen

5       The polypeptide having the amino acid sequence of the 265-586th in SEQ ID NO: 1 may be prepared by, for example, cleaving NAIP cDNA having the nucleotide sequence of SEQ ID NO: 2 with a restriction enzyme to obtain a DNA fragment containing the nucleotide sequence of the 1056-2049th, and expressing the DNA fragment in an appropriate host-vector system. The 10 polypeptide having the amino acid sequence of the 841-1052th in SEQ ID NO: 1 may be prepared by expressing a DNA fragment having the nucleotide sequence of the 2812-3447th in SEQ ID NO: 2 in an appropriate host-vector system.

15       Alternatively, polypeptide having a partial sequence (10-20 amino acids) of the amino acids sequence of the 256-586th or the 841-1052nd region in SEQ ID NO: 1 may be prepared. In this case, by using polypeptides of different sequences, hybridomas each producing monoclonal antibody of different epitope can be obtained.

20       These polypeptides may be also used in a form of a fusion protein in which the polypeptide is fused with other proteins (e.g., glutation-S-transferase: GST). Use of such fusion proteins is especially preferable in terms of facilitating and ensuring the separation process of the targeted protein from the expressed product of the host-vector system and the screening process (described below) of the hybridoma.

25       It should be noted that the polypeptide may be that having amino acid sequence in which at least one amino acid residue is deleted or substituted or added in amino acid sequence of the 256-586 or a part in SEQ ID NO: 1.

30       b) Immunization of animals

      As the animals to be immunized, mammals used in the known hybridoma preparation methods can be employed. Specific examples of the

5 animals include mice, rats, goats, sheep, cows and horses. However, in terms of availability of myloma cells to be fused with the isolated antibody-producing cells, it is preferable to use mice or rats as the animals to be immunized. There is no particular restriction on the strains of mice and rats  
10 actually used. In the case of mice, examples of strains thereof which can be used include A, AKR, BALB/c, BDP, BA, CE, C3H, 57BL, C57BR, C57L, DBA, FL, HTH, HT1, LP, NZB, NZW, RF, RIII, SJL, SWR, WB, 129. In the case of rats, examples of strains thereof that can be used include Low, Lewis, Spraque, Daweley, ACI, BN, Fisher. Among them, if the suitability in being fused with the myeloma cells described below is considered, the "BALB/c" strain of mice and the "Low" strain of rats are especially preferable as the animals to be immunized. It is preferable that the mouse or rat is 5-12 week old when it is immunized.

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15 The immunization of the animal can be carried out by subcutaneously or intraperitoneally dosing the polypeptide solution as an immunogen, into the animal. The dosing schedule of the antigen varies depending on the types of the subject animal or the differences between the individual animals. In general, the antigen is preferably dosed totally 2-6 times with 1-2 weeks of the interval between doses. The amount of the antigen to be dosed also varies depending on the types of the animal and the differences between the individual animals. In general, the amount of the antigen to be dosed is approximately 10-100 µg/µl.

### c) Fusion of cells

20 1-5 days after the final immunization in the aforementioned dosing schedule, spleen cells or lymphatic cells containing the antibody-producing cells are steriley collected from the immunized animal. The separation of the antibody-producing cells from the spleen cells or the lymphatic cells can be carried out according to the known methods.

25 Next, the antibody-producing cells are fused with myeloma cells. There is no particular restriction on the myeloma cells to be used, and those appropriately selected from the known cell lines may be used. However, in

consideration of the convenience at the time of selecting hybridomas from the fused cells, it is preferable to employ a HGPRT (Hpxoxanthine-guanine phosphoribosyl transferase) defective line for which a selection procedure has been established. Specific examples thereof include: X63-Ag8(X63), NS1-  
5 Ag4/1(NS-1), P3X63-Ag8.U1(P3UI), X63-Ag8.653(X63.653), SP2/0-  
Ag14(SP2/0), MPC11-45.6TG1.7(45.6TG), FO, S149/5XXO.BU.1, which are derived from mice; 210.RSY3.Ag.1.2.3(Y3) derived from rats; and U266AR(SKO-007), GM1500 · GTG-A12(GM1500), UC729-6, LICR-LOW-HMy2(HMy2), 8226AR/NIP4-1(NP41), which are derived from human.

10 The antibody-producing cells may be fused with the myeloma cells in an appropriate manner, according to the known method, under a condition in which the survival rate of the cells does not drop to such an extremely low level. Examples of such methods include a chemical method in which the antigen-producing cells are mixed with the myeloma cells in a polymer (e.g., polyethylene glycol) solution of a high concentration, a physical method in which electric stimulation is utilized, and the like.

15 E20 The selection of the fused cells from the non-fused cells is preferably carried out according to the known HAT (Hpxoxanthine/ Aminopterin/ Thymidine) selection method. This method is effective when fused cells are obtained by using myeloma cells of a HGPRT defective line that is not viable under the presence of aminopterin. That is, by cultivating fused cells and cells which have not been fused in a HAT culture, only the fused cells that is resistant to aminopterin are selectively remained and allowed to reproduce.

25 d) Screening of hybridoma

The screening of the hybridoma which produce the targeted monoclonal antibody can be performed by the known EIA (Enzyme Immunoassay), RIA (Radio Immunoassay), fluorescent antibody methods and the like. When a fused protein is employed as the immunogen, the 30 hybridoma can be screened more reliably by carrying out the aforementioned screening methods for the protein which is the partner of the fusion, as well.

By conducting such a screening process, hybridomas respectively

producing monoclonal antibodies having different epitope domains are obtained. Accordingly, the monoclonal antibodies of the present invention include all of the plural types of monoclonal antibodies respectively produced by the hybridomas prepared by the method described above.

5 After the screening process, the hybridomas are then subjected to cloning by the known methods such as the methylcellulose method, the soft agarose method and the limiting dilution method, so that the hybridomas can be used for producing the antibodies.

10 The hybridomas obtained by the aforementioned method can be stored in the frozen state in liquid nitrogen or in a freezer in which the temperature is no higher than -80°C.

## 2. Production of the monoclonal antibodies and purification thereof

The monoclonal antibodies that specifically recognize NAIP can be obtained by cultivating, according to the known method, the hybridomas prepared as described in the paragraph 1 above.

15 The cultivation may be conducted, for example, in the culture having the same composition as that used in the cloning method described above. Alternatively, in order to produce a large amount of the monoclonal antibodies, it is acceptable to inject the hybridoma intraperitoneally to a mouse and collect the monoclonal antibody from the ascites of the animal.

20 The monoclonal antibody obtained in such a manner can be purified by the methods including the ammonium sulfate salting out method, the gel filtration method, the ion-exchange chromatography method, the affinity chromatography method and the like.

Next, the NAIP assay method of the present invention will be described hereinafter.

30 In the first assay method, a solution of the marker-labeled anti-NAIP monoclonal antibody (M-mAb) is contacted with a sample containing NAIP so

that the marker-labeled monoclonal antibody is bound with NAIP, and the bound structure (M-mAb: NAIP) are separated. As the means for separation, any known methods including the chromatography method, the salting out method, the alcohol precipitation method, the enzyme method, the solid 5 phase method and the like may be employed. In a case in which an enzyme is used as the marker, a substrate that develops a color as a result of decomposition by the enzyme activity is added. In this case, the activity of the enzyme is measured by optically measuring the amount of the decomposed substrate and the activity of the enzyme is converted into the 10 amount of bound antibody, so that the amount of NAIP is calculated on the basis of the comparison of the obtained amount of bound antibody with the reference value. In a case in which a radioactive isotope is used as the marker, the amount of the radioactive rays emitted from the radioactive isotope is measured by a scintillation counter or the like. In a case in which a fluorescent colorant is used as the marker, a device in which a fluorescent microscope is incorporated can measure the magnitude of fluorescence.

In the second assay method, two types of antibodies whose epitope domains for NAIP are different from each other (the primary antibody and the secondary antibody) are used. Specifically, at first the primary antibody (Ab 1) is contacted with a sample containing NAIP so that the primary antibody and NAIP are bound with each other. The bound structure (Ab I: NAIP) is bound with the secondary antibody that has been marker-labeled (M-Ab II), and the signal strength of the marker in the bound structure of the three 25 components (Ab I: NAIP: M-Ab II) is measured. Optionally, in order to make the signal stronger, it is acceptable to allow the bound structure (Ab I: NAIP) to be bound, at first, with secondary antibody that is not marker-labeled and then allow the secondary antibody to be bound with the marker. Such bonding of the secondary antibody with a marker-labeled molecular can be 30 effected, for example, by using the secondary antibody with biotin and the marker with avidin. Further, it is also acceptable that an antibody (the tertiary antibody) that recognizes a portion of the secondary antibody (e.g., Fc

domain) is marker-labeled, so that the tertiary antibody is bound with the secondary antibody (II). The anti-NAIP monoclonal antibodies of the present invention may be used for both the primary antibody and the secondary antibody. Alternatively, the anti-NAIP polyclonal antibody (the anti-serum of 5 the animals immunized by the aforementioned polypeptide, for example) may be used for one of the primary antibody and the secondary antibody.

Although this second method can be carried out either in the liquid phase or on the solid phase, it is preferable to carry out the method on the 10 solid phase, in order to make the assay of extremely small amounts and the operation as a whole easier. More specifically, the solid phase method includes the steps of: providing the primary antibody on a resin plate or the like so that the primary antibody is immobilized; allowing the antibody on the solid state to be bound with NAIP; washing off the NAIP which is not bound to the antibody; allowing the bound NAIP remaining on the plate to be bound with the secondary antibody; and measuring the signal strength of the secondary antibody. This is what is called the "sandwich method", and widely used as "ELISA" (enzyme linked immunospecific assay) when an enzyme is used as the marker.

In the methods described above, there is no particular limitation on the enzyme used as the marker, as long as the turn over number of the enzyme is relatively large, the enzyme is stable after being bound with the antibody, the enzyme specifically acts on the substrate so that the substrate 25 develops a color, and other required conditions are satisfied. Examples of the enzyme include the enzymes commonly used for EIA, such as peroxydase,  $\beta$ -galactosidase, alkali-phosphatase, glucoseoxydase, acetylchlorine-esterase, glucose-6-phosphorylation dehydrogenase, malic acid dehydrogenase and the like. Further, enzyme inhibitors and coenzymes may also be used. Bonding 30 of these enzymes with the monoclonal antibody can be carried out according to the known method which employs a cross-linking agent such as maleimide compounds. As the substrate, any suitable known compounds may be used,

depending on the types of the enzyme that is actually used. In a case in which peroxydase is used as the enzyme, 3, 3' 5, 5'-tetramethylbenzidine may be used as the substrate. In a case in which alkli-phosphatase is used as the enzyme, para-nitrophenol or the like may be used as the substrate.

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In a case in which a radioactive isotope is used as the marker, examples of the radioactive isotope include those used in the standard RIA process such as  $^{125}\text{I}$  and  $^3\text{H}$ . Examples of the fluorescent colorants include those used in the standard fluorescent antibody method such as fluorescence 10 isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC).

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The assay kits of the present invention may be used for the "sandwich method" in which the aforementioned second assay method is carried out on the solid phase. Such kits of various types are commercially available in accordance with the types of the components to be assayed. The assay kits of the present invention may be basically constituted of various components used in known and commercially available kits, except that the aforementioned anti-NAIP monoclonal antibody and/or the anti-NAIP polyclonal antibody is used as the antibodies. In addition, the assay kits of the present invention including the components described above may be provided with a washing solution for washing off the NAIP which has not been bound and/or the secondary antibody which has not been bound.

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### Examples

The present invention will be described in detail by examples hereinafter. It should be noted, however, that the present invention is not limited to any of these examples.

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Example 1: Production of the monoclonal antibodies

## (1) Preparation of the immunogen

The 1056-2049th region of NAIP cDNA of which nucleotide sequence is shown in SEQ ID NO: 2 was amplified, and the DNA fragment (NAIP.256-586) was inserted at the EcoR I site of pGEX-3X (by Pharmacia Co.). After confirming the nucleotide sequence, the host Escherichia coli BL21 (DE3) pLysS was transformed by this recombinant vector pGEX-3X(NAIP.256-586) and cultivated in the LB medium for 5 hours at 30°C. Thereafter, IPTG was added to the medium and the cultivation was continued at 20°C for 3 hours. The bacteria was separated by centrifuging, dissolved into the dissolving solution (PBS, Triton X-100), frozen at -80°C and melted, and then subjected to ultrasonic destruction. The product was centrifuged at 1000 × g for 30 minutes, the supernatant was introduced to a glutathione sepharose 4B column so as to pass through it, whereby fusion protein GST-NAIP(256-586) was obtained.

In addition, the 2812-3447th region of NAIP cDNA of which nucleotide sequence is shown in SEQ ID NO: 2 was amplified, and the DNA fragment (NAIP841-1052) was inserted at the BamHI-Sall site of pGEX-4X-3 (by Pharmacia Co.). Thereafter, the same method as described above was repeated, thereby obtaining the fusion protein GST-NAIP(841-1052).

## (2) Immunization of the animal

50 µg/µl of each of the fusion proteins obtained in the aforementioned (1) was dosed to a Balb/c mouse, intraperitoneally, as the initial immunization. The second immunization was performed 2 weeks after  
25 the initial immunization, and immunization was conducted totally six times with one-week interval. At the initial immunization, the fusion protein was dosed in a state in which Freund complete adjuvant of the equal amount was mixed thereto. At the second to fifth immunization, the fusion protein was dosed in a state in which Freund incomplete adjuvant was mixed thereto. At  
30 the final immunization, only the fusion protein solution was dosed.

### (3) Fusion of cells

The spleen cells were steriley isolated three days after the final immunization. The collected spleen cells and the myeloma cell line SP2/0-Ag14 derived from mice were mixed and then subjected to the fusing treatment by using polyethylene glycol #4000. The obtained cells were planted on a 96-hole plate, and the fused cells were selected by the HAT culture.

#### (4) Screening

An ELISA plate on which the NAIP polypeptide used as the immunogen was provided on the solid state and an ELISA plate on which GST was provided in the solid state were prepared. Clones that did not react to the GST plate but reacted only to the NAIP plate were selected and subjected to screening. Next, among the supernatants of the cultures of respective hybridomas, the wells reacted to the NAIP polypeptide were regarded as positive. The cloning of the hybridomas was carried out by using the positive wells in the limiting dilution method. The screening process was repeated for the cultures of the hybridomas that were supposed to have only single-type clones, whereby a plurality of hybridomas was obtained. Among these plural hybridomas, hybridomas 656-1, 656-2 and hnmc841 were deposited to National Institute of Bioscience and Human-Technology. The deposit Nos. of these hybridomas are FERM BP-6919 (hybridoma 656-1), FERM BP-6920 (hybridoma 656-2) and FERM BP-6921 (hybridoma hnmc841), respectively.

## (5) Production of the monoclonal antibodies

Three types of the hybridomas obtained as described above were dosed to a Balb/c mice, intraperitoneally, and the ascites containing the monoclonal antibody was collected after one week. From the collected ascites, the three types of monoclonal antibodies hnmc365, hnmc381 and hnmc841 were purified by using an affinity column in which protein G was used.

It was confirmed that the monoclonal antibody hnmc365, produced by hybridoma 656-1 which had been prepared by using fusion protein GST-NAIP(256-586) as the immunogen, belongs to the subclass IgG1 and the epitope thereof is the amino acid sequence of the 254-368th region in SEQ ID NO: 1. It was also confirmed that the monoclonal antibody hnmc381 produced by hybridoma 656-2 belongs to the subclass IgG2b and the epitope thereof is the amino acid sequence of the 373-387th region in SEQ ID NO: 1. Further, it was confirmed that the monoclonal antibody hnmc841, produced by the hybridoma hnmc841 which had been prepared by using fusion protein GST-NAIP(841-1052) as the immunogen, belongs to the subclass IgG1 and the epitope thereof is the amino acid sequence of the 841-1052nd region in SEQ ID NO: 1.

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15 Example 2: Production of the polyclonal antibody

A rabbit (Japanese White Rabbit) was immunized by the standard method, by using as the immunogen the fusion protein GST-NAIP(256-586) prepared in a manner similar to that of Example 1 (1). The anti-serum was then separated, and the polyclonal antibody was purified by a sepharose 4B column in which the aforementioned fused proteins were bonded.

Example 3: Production of ELISA kit

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(1) Primary antibody-immobilized plate

A solution (20 µg/ml) of the anti-NAIP monoclonal antibody hnmc365 produced in Example 1 was dissolved into 10 mmol/l of potassium phosphate buffer (pH 7.5) containing 150 mmol/l of sodium chloride and 1 g/l of sodium azide. 50 µl of this solution was pipetted into each hole of a 96-hole plate for ELISA. The plate was stored at 4°C for 16 hours. Thereafter, the plate was washed with 10 mmol/l potassium phosphate buffer (pH 7.5) containing 150

mmol/l sodium chloride, whereby the plate on which the anti-NAIP monoclonal antibody was immobilized was produced.

(2) Biotinylated secondary antibody

5        0.01 mmol of biotin-amidecaproic acid N-hydroxysuccinic imide ester dissolved into N, N-dimethylformamide was added to 10 mg of the anti-NAIP polyclonal antibody produced in Example 2. The mixture was stored at 25°C for 3 hours and then subjected to dialysis for 16 hours in 50 mmol/l potassium phosphate buffer (pH 7.4), whereby the biotinylated anti-NAIP 10 polyclonal antibody was produced.

(3) Marker to be bound to the secondary antibody

A solution of horse radish peroxidase-labeled streptoavidin was diluted to the concentration of 0.5 µg/ml with 10 mmol/l potassium phosphate buffer (pH 7.2) containing 150 mmol/l sodium chloride and 1 g/L casein, whereby the marker solution was obtained.

Example 4: NAIP assay

(1) Method of operation

Sample solutions containing the purified NAIP at different concentrations were diluted with 10 mmol/l potassium phosphate buffer (pH 7.2) containing 150 mmol/l sodium chloride. 50 µl of each of the diluted 25 solutions was pipetted into each hole of the plate on which the primary antibodies had been provided in the solid state prepared in Example 3 (1). The plate was stored at 37°C for 1 hour and then washed off with 10 mmol/l potassium phosphate buffer (pH 7.2) containing 150 mmol/l sodium chloride.

Next, the biotinated anti-NAIP polyclonal antibody of Example 3 (2) 30 was diluted to the concentration of 0.5 µg/ml with 10 mmol/l potassium phosphate buffer (pH 7.2) containing 150 mmol/l sodium chloride and 1 g/l casein. 100 µl of each of the diluted solutions was pipetted into each hole of

the aforementioned plate. The plate was stored at 37°C for 1 hour and then washed off with 10 mmol/l potassium phosphate buffer (pH 7.2) containing 150 mmol/l sodium chloride.

As the final step, 100 µl of the solution of horse radish peroxydase-labeled streptoavidin prepared in Example 3 (3) was pipetted into each hole of the aforementioned plate. The plate was stored at 37°C for 1 hour and then washed off with 10 mmol/l potassium phosphate buffer (pH 7.2) containing 150 mmol/l sodium chloride.

10 (2) Color-developing reaction and measurement of absorbance

3,3',5,5'-tetramethylbenzidine was dissolved into N,N-dimethylformamide so that the concentration of 3,3',5,5'-tetramethylbenzidine was 50 mmol/l. The obtained solution was diluted to 1/100 with 100 mmol/l sodium acetate buffer (pH 5.5) and then filtered by a filtering paper. 0.1 ml of aqueous hydrogen peroxide (10 g/l) was added to 10 ml of the solution, whereby the color developing solution was obtained. 50 µl of the color developing solution was pipetted into each hole of the aforementioned plate. The plate was stored at 30°C for 30 minutes. Thereafter, 50 µl of sulfuric acid (2 mol/l) was pipetted into each hole of the plate, so that the reaction stopped. Absorbance was then measured at 450 nm.

(3) Results

Fig. 1 is a graph that shows the relationship between the concentration of the purified NAIP in the sample solution and the absorbance measured by the aforementioned method. The concentration of NAIP in the sample was measurable because the values thereof resided within the measurable range of 4 ng/ml to 20 ng/ml.

From the results, it was confirmed that, if the NAIP concentration is unknown for a sample, the concentration of NAIP of the sample can be accurately assayed on the basis of the absorbance thereof by utilizing, for example, the measurement results as shown in Fig. 1 as the reference line.

**Example 5: Western Blot****5 (1) Preparation of sample for SDS gel electrophoresis**

Mononuclear cells were separated from 10 ml of normal human peripheral blood by using Ficoll Paque PLUS (by Amasham-Pharmacia Co.). The obtained mononuclear cells were fixed by 5-10 % trichloroacetic acid and then were separated by centrifuging. The separated cells were dissolved into a Tris buffer containing lithium dodesyl sulfate (2%), urea (8M), DTT (1%), and Triton X-100 (1%).

**(2) Western Blot**

Using the aforementioned sample carried out SDS gel electrophoresis, and the result was transferred to a PVDF film. The PVDF film on which the transfer had been done was treated overnight at 4°C with TBS containing skimmed milk (10%) and Tween 20 (0.05%). The PVDF film was then washed with TBS (TBST) containing Tween 20 (0.05%). Each antibody was diluted with TBST in an appropriate manner and allowed to react at the room temperature for 2 hours. Then, after washing with TBST, peroxydase-labeled anti-rabbit Ig antibody or anti-mouse Ig antibody (by Amasham-Pharmacia Co.) was added for reaction that proceeded at the room temperature for 1 hour. After washing with TBST, the treatment with the ECL PLUS reagent (by Amasham-Pharmacia Co.) and exposure onto an X-ray film followed, whereby signals were obtained.

**(3) Results**

The results are shown in Fig. 2. In all of the three types of blots in which the monoclonal antibodies were used, signals of 160 kDa which had been observed for the anti-NAIP polyclonal antibody were detected.

From the aforementioned results, it was confirmed that the monoclonal antibodies hnmc365, hnmc381 and hnmc841 prepared in

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Example 1 are monoclonal antibodies that specifically recognize NAIP and thus detection of NAIP is possible by using these monoclonal antibodies.

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### **Industrial Applicability**

As described above in detail, the invention of the present application allows simple but accurate assay of human apoptosis inhibitory protein (NAIP) present in a sample isolated from an organism. The present invention will facilitate better understanding of the mechanism of patients developing symptoms of various apoptosis-related diseases, better diagnosis of the danger of developing the diseases, prevention of the development of the diseases or reduction of the severity of the diseases, and development of the medical technique and medicines for treatment.

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